Glyoxalase and Methylglyoxal in Thiamine-Deficient Rats

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Although it is by no means certain that methylglyoxal is the natural substrate of glyoxalase, it seems reasonable to seek this substance in the animal under various conditions of metabolism, in spite of the difficulties of detecting it in the presence of the highly active glyoxalase. As pointed out by Hopkins & Morgan (1945) the absence of satisfactory evidence for the occurrence of a substrate for glyoxalase in the cells and tissues studied has been disappointing to those interested in the enzyme. However, many reports suggest that conditions permitting the formation of methylglyoxal exist in the cells. Thus Pinkus (1898) observed the formation of methylglyoxal-bis-phenylhydrazone on treating glucose with dilute sodium hydroxide in the presence of phenylhydrazine. Neuberg & Kobel (1928) reported that methylglyoxal could be isolated from the products of the fermentation of hexose diphosphate by extracts of dried bottom yeast in the presence of toluene. Tönniessen & Fischer (1926) reported that methylglyoxal is formed from hexose diphosphate by mixed preparations of pig muscle and pancreas. They were unable to obtain methylglyoxal when glucose or glycogen replaced the hexose diphosphate. A significant point in this work is the use of pancreas which, by the presence of its antiglyoxalase, retards the further conversion into lactic acid of methylglyoxal produced from hexose diphosphate. Ariyama (1928) repeated this work using 'Pancreatin' as a source of antiglyoxalase. The present author (Salem, 1950) confirmed the conversion of hexose diphosphate into methylglyoxal in liver slices using either pancreatic antiglyoxalase, which inhibits glyoxalase itself, or kidney antiglyoxalase which destroys the coenzyme glutathione. Evidence was obtained for the enzymic nature of the system responsible for this latter reaction.

Early attempts to introduce methylglyoxal into the scheme of carbohydrate metabolism had assumed the non-toxicity of this substance. However, the demonstration by Stöhr (1932) that it was toxic to rats, although if fed at the correct level it increased the liver glycogen, and the finding of Kun (1950) that it inhibited the succinic oxidase system has required revision of this assumption.

Previous workers have tried to link the effect of thiamine with the supposed importance of methylglyoxal in carbohydrate metabolism. Thus Findlay (1921) reported that the livers of pigeons suffering from polyneuritis were deficient in glyoxalase, and an increase in the glyoxalase content occurred on feeding thiamine. However, thiamine did not act as a coenzyme for glyoxalase. In 1931 Vogt-Møller described thiamine deficiency as methylglyoxal intoxication, caused by depriving the tissues of glyoxalase. In keeping with this, Geiger & Rosenberg (1933) detected methylglyoxal in the urine of polyneuritic dogs and in the urine and cerebrospinal fluid of infants suffering from acute toxic dyspepsia, which is thought to be caused by thiamine deficiency. However, Johnson (1936) was unable to detect any preformed methylglyoxal in the tissues of polyneuritic pigeons.

Most attempts to demonstrate the formation of methylglyoxal under biological conditions were carried out before the discovery of glutathione as the coenzyme of glyoxalase (Lohmann, 1932), and before the recent work on the properties of the enzyme and its inhibitors (Crook & Law, 1950, 1952; Racker, 1950, 1951; Salem, 1950). This, together with the apparent contradiction between the work of Johnson (1936) and of Findlay (1921) and Geiger & Rosenberg (1933), suggested the desirability of further experiments to investigate the possible relationship between the glyoxalase system and thiamine deficiency.

MATERIALS AND METHODS

Rats. Two series of experiments were carried out, one with adult males and the other with newly weaned male animals. The adults were albinos of 120–150 g. weight, and were being used for thiamine assay. The animals were kept separately in Hopkins cages with screened floors to exclude coprophagy, and urines were collected separately from faeces by the use of a separator. The animals were given a synthetic diet deficient in thiamine, but complete in all other known respects. One or two drops of cod-liver oil were fed daily to each rat.

Marked loss of appetite, rapid loss of weight with signs of marasmus, and difficulty in the use of the limbs followed by paralysis, were shown by the adults after 4–5 weeks on the deficient diet. The newly weaned rats showed the same symptoms after 3 weeks. Control animals were kept on a normal diet containing thiamine.

Urines. The urine of each rat was collected separately, concentrated by distillation under reduced pressure (40–50 mm.), and the pH adjusted to 7.

Measurement of glyoxalase activity. Glyoxalase activity was measured by the method of Hopkins & Morgan (1945). using 3 mg. of methylglyoxal of 85% purity, 0·2 mg.

glutathione, 0·4 ml. of 0·2M-$NaHCO_3$ and 0·4 ml. enzyme solution in Warburg manometers.

Estimation of methylglyoxal. This was done by a modification previously employed (Salem, 1950) of Woodward's (1935) enzymic method for the determination of glutathione. Other methods depend on the reducing power of methylglyoxal (Ariyama, 1928) or the precipitation of the bis-phenylhydrazone (Vogt-Møller, 1931). The specificity of the enzymic method is much higher than these, and was, therefore, preferred. A 1:5 (w/v) aqueous extract of acetone-dried rat liver was purified by the method of Hopkins & Morgan (1945) to stage III. The purified enzyme solution (0·4 ml.) was used under the same conditions as for the determination of glyoxalase, but with varying amounts of methylglyoxal. A blank without methylglyoxal was used for correcting the experimental values. The total CO_2 produced after completion of the reaction (approx. 10 min.) was plotted against the amount of methylglyoxal taken. This formed a standard curve which was a straight line over most of the range (Fig. 1). From it the methylglyoxal content of an unknown solution could be determined by measuring the CO_2 output in the presence of the standard enzyme solution.

Hexose diphosphate. The hexose diphosphate used was the sodium salt prepared from the calcium salt by treatment with sodium oxalate.

RESULTS

Methylglyoxal and thiamine deficiency

The urine of thiamine-deficient adult rats was collected every 2 days after the symptoms of deficiency had appeared. After evaporation under reduced pressure, 1 ml. of the concentrate was assayed for methylglyoxal using liver glyoxalase. Controls were run containing 3 mg. of methylglyoxal and also a blank without urine or methylglyoxal. The experiments were carried on for 20 min. and the volume of CO_2 evolved is shown in Table 1.

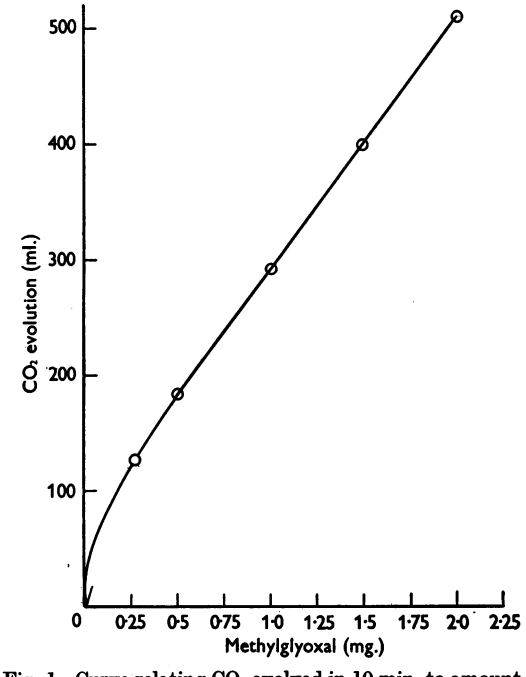

Fig. 1. Curve relating CO_2 evolved in 10 min. to amount of methylglyoxal in the presence of reduced glutathione and glyoxalase.

The experiments were repeated on male newly weaned rats in which the symptoms of thiamine deficiency were shown earlier than in adults. Table 1 shows that the CO_2-evolving material was present in the urine of these thiamine-deficient rats to about the same extent as in that of the adults. Both sets of experiments showed that no CO_2 is evolved from the urine of normal animals in the presence of glyoxalase.

A group of thiamine-deficient animals were given orally 0·3 mg. of thiamine hydrochloride each day and their diet was changed to a normal diet. After a week these animals recovered completely and began to gain weight. The urine of these recovered rats was tested for methylglyoxal as before. Table 1 shows that the CO_2-evolving material in the urine had now disappeared.

Glyoxalase and thiamine deficiency

Glyoxalase was extracted from the livers of normal and thiamine-deficient animals by grinding with sand in a mortar and extracting with 5 parts of water. The extraction was carried on with occasional stirring for 30 min. at room temperature. The mixture was centrifuged, and the supernatant liquid withdrawn and preserved in the refrigerator. Both methylglyoxal and phenylglyoxal were used as substrates, conditions otherwise being those shown in the methods section. The results are shown in Fig. 2, from which it is seen that glyoxalase activity on both substrates is very low in liver extracts from rats on diets deficient in thiamine, compared with those from normal livers. These experiments were

Table 1. *Methylglyoxal content of the urine of normal and thiamine-deficient male rats as estimated by the evolution of* CO_2 *from bicarbonate on the addition of glyoxalase + glutathione*

Values are for 24 hr. output

Source of urine	μl. CO_2/ 20 min.*	No. of animals used
Thiamine-deficient weaned young rats	88±6	4
Thiamine-deficient adults	132±3	5
Deficient adults after treatment with thiamine	8±1	3
Normal adults	6	—
Blank value (no urine added)	6	—

* Mean value obtained from several replicate determinations on each sample of urine. Under the same conditions 3 mg. of methylglyoxal (85% purity) yielded 359±12 μl. CO_2.

repeated many times with similar results. A group of thiamine-deficient rats were given thiamine orally as before, and the livers subsequently assayed for glyoxalase. Fig. 2 shows that the glyoxalase activity of the recovered rats had completely regained the normal level.

Methylglyoxal formation in thiamine-deficient livers

The production of methylglyoxal from hexose diphosphate was investigated in liver suspensions from normal animals, from animals deficient in thiamine, and from rats which had been deficient in thiamine, but which had recovered after receiving thiamine in the diet. Controls were also done with normal animals in which kidney antiglyoxalase was used to inhibit the glyoxalase, as described by Ariyama (1928).

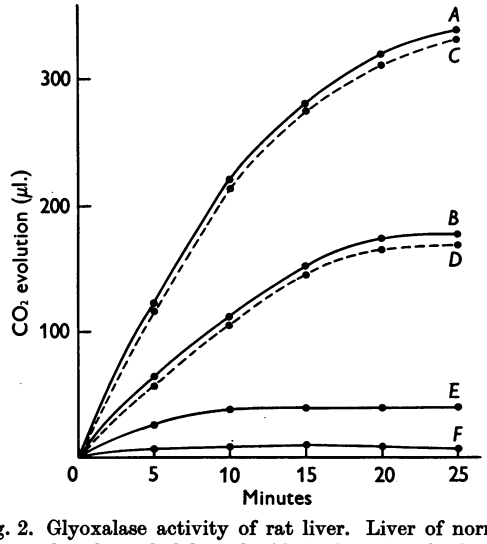

Fig. 2. Glyoxalase activity of rat liver. Liver of normal animal with methylglyoxal added (*A*) and with phenylglyoxal (*B*). Liver of animal recovered from thiamine deficiency with methylglyoxal added (*C*) and with phenylglyoxal (*D*). Liver of thiamine-deficient animal with methylglyoxal added (*E*) and with phenylglyoxal (*F*). Conditions as in methods section.

The liver preparation was made by grinding the tissue finely in a mortar. The material was suspended in 1 ml. of borate:boric acid buffer, pH 7, hexose diphosphate was added with toluene as an antiseptic, and incubated at 37° for 24 hr. At the end of this time the proteins were precipitated by the addition of 0·5 ml. of 10% (w/v) sulphosalicylic acid and were centrifuged off. The supernatant fluid was adjusted to pH 7·0 with NaOH and the volume adjusted to 4 ml. 1 ml. samples were taken for the estimation of methylglyoxal.

The results are shown in Table 2, from which it can be seen that methylglyoxal is produced by suspensions of livers from thiamine-deficient animals and by suspensions of normal livers in which the glyoxalase activity has been inhibited, but not by livers of normal rats or of animals which have recovered from thiamine deficiency. The blanks show that no methylglyoxal is formed in the absence of hexose diphosphate.

DISCUSSION

The work reported here confirms the earlier observations of Findlay (1921) and of Geiger & Rosenberg (1933) that animals deprived of thiamine excrete methylglyoxal in the urine. These earlier workers depended upon the isolation of the phenylosazone of methylglyoxal, whereas here the specific enzyme has been employed. This has been possible because of the absence both of the enzyme and its substrate from normal urine, as first noted by Dakin & Dudley (1913).

The appearance of methylglyoxal in the urine during thiamine deficiency appears to be due to the lack of glyoxalase activity in the liver. This enzyme normally ensures the conversion of methylglyoxal into lactic acid. The lack of glyoxalase in the liver and the excretion of methylglyoxal in the urine appear to be a direct result of the thiamine deficiency, since restoration of this substance to the diet immediately restores the glyoxalase activity of the liver and abolishes the excretion of methylglyoxal.

Table 2. *Production of methylglyoxal from hexose diphosphate*

The reaction mixtures contained 0·5 g. liver pulp, 1·0 ml. borate buffer, pH 7·0, 2·0 ml. 0·5% hexose diphosphate and 0·1 ml. toluene. In *B*, 0·5 ml. of 1:5 fresh kidney extract was present. In other flasks this was replaced by 0·5 ml. water. Flask *E* contained no hexose diphosphate. Incubation at 37° for 24 hr.

	Methylglyoxal (mg.)				
Expt.	*A* Thiamine-deficient	*B* Normal + antiglyoxalase	*C* Normal	*D* Recovered from thiamine deficiency	*E* Blank
1	1·48	1·60	0·11	0·16	0
2	1·40	1·60	0·06	0·06	0
3	1·24	1·38	0·09	0·09	0
4	1·64	1·52	0·12	0·07	0

It is not clear whether the lack of glyoxalase activity in the livers of thiamine-deficient rats is due to the disappearance of one or both of the two components of the enzyme (cf. Crook & Law, 1952), or whether it is due to lack of the coenzyme, reduced glutathione. Experiments are in progress to clarify this point.

Since thiamine deficiency causes a derangement of carbohydrate metabolism at the level of pyruvate oxidation, and at the same time causes methylglyoxal to be excreted, it might be expected that methylglyoxal would be formed from carbohydrate intermediates earlier in the series than pyruvate. Some indication that this may be so comes from the demonstration that hexose diphosphate can give rise to methylglyoxal in the livers of thiamine-deficient rats. That the demonstrable formation of methylglyoxal is due to the absence of glyoxalase activity and not to some other derangement of carbohydrate metabolism is shown by the fact that normal livers also produce methylglyoxal from hexose diphosphate when their glyoxalase is inhibited by removing its coenzyme. This would suggest that methylglyoxal formation normally occurs in liver and that it is not usually detected because of its further conversion into lactic acid, a view held some years ago but since abandoned.

The abandonment of this concept was due to two chief causes: first, the outstanding importance of the phosphorylated intermediates between hexose diphosphate and pyruvate; and secondly, the claim that the lactic acid produced by glyoxalase was of the wrong optical configuration (Lohmann, 1932; Levene & Meyer, 1913). However, when the published figures are examined it will be found that, except in the case of *Lactobacillus arabinosis* where a fairly pure D-lactic acid is formed, the most that has been demonstrated is that glyoxalase produces a mixture of the two optical isomers in which the D form predominates. Moreover, the lactic acid in these experiments has invariably been isolated as its zinc salt which Purdie & Walker (1892) have shown to lead to partial resolution. In addition, Cori & Cori (1928) and Craig (1946) have shown with rats and dogs, respectively, that, although the L form is preferentially utilized, the D form is quite rapidly assimilated.

Thus it would seem feasible to reintroduce methylglyoxal into the general scheme of carbohydrate metabolism, if only as a minor side branch. Methylglyoxal is a toxic substance (Stöhr, 1932; Kun, 1950), and the role of glyoxalase may be merely that of detoxication. The wide distribution of the enzyme in all types of tissue from species of all phyla is in keeping with a role of this kind. As Vogt-Møller (1931) has pointed out, the symptoms of thiamine deficiency may well be those of methylglyoxal intoxication. However, this remains to be established, although the present findings are not incompatible with such a concept.

SUMMARY

1. Methylglyoxal is present in the urine of thiamine-deficient rats.

2. Restoration of thiamine to the diet causes the disappearance of the methylglyoxal from the urine.

3. The glyoxalase activity of the livers of thiamine-deficient rats is much lower than that of normal animals, and is restored to normal by including thiamine in the diet.

4. Methylglyoxal is produced by liver suspensions from thiamine-deficient rats when hexose diphosphate is added. Methylglyoxal does not accumulate in normal liver suspensions because of the presence of glyoxalase in the latter.

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